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ARTICLE

Effects of anti-inflammatory drugs on the expression of tryptophan-metabolism genes by human macrophages

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Abstract

Several lines of evidence link macrophage activation and inflammation with (monoaminergic) nervous systems in the etiology of depression. IFN treatment is associated with depressive symptoms, whereas anti-TNF α therapies elicit positive mood. This study describes the actions of 2 monoaminergic antidepressants (escitalopram, nortriptyline) and 3 anti-inflammatory drugs (indomethacin, prednisolone, and anti-TNF α antibody) on the response of human monocyte-derived macrophages (MDMs) from 6 individuals to LPS or IFN α . Expression profiling revealed robust changes in the MDM transcriptome (3294 genes at $P < 0.001$) following LPS challenge, whereas a more limited subset of genes (499) responded to IFN α . Contrary to published reports, administered at nontoxic doses, neither monoaminergic antidepressant significantly modulated the transcriptional response to either inflammatory challenge. Each anti-inflammatory drug had a distinct impact on the expression of inflammatory cytokines and on the profile of inducible gene expression—notably on the regulation of enzymes involved in metabolism of tryptophan. Inter alia, the effect of anti-TNF α antibody confirmed a predicted autocrine stimulatory loop in human macrophages. The transcriptional changes were predictive of tryptophan availability and kynurenine synthesis, as analyzed by targeted metabolomic studies on cellular supernatants. We suggest that inflammatory processes in the brain or periphery could impact on depression by altering the availability of tryptophan for serotonin synthesis and/or by increasing production of neurotoxic kynurenine.

KEYWORDS

anti-inflammatory, depression, inflammatory signaling, kynurenine, macrophage, monoaminergic, transcriptomics, tryptophan

1 | INTRODUCTION

Autoimmune and inflammatory diseases are commonly associated with mood disorders and several lines of evidence indicate inflammation may give rise to or exacerbate them.^{1,2} Studies of animals exposed to proinflammatory challenges, ranging from LPS administration

to social defeat, reveal that activation of the peripheral innate immune system causes a depression-like syndrome of illness behavior: social withdrawal, reduced mobility/energy, sleep disturbance, weight loss, and anhedonia.^{3–5} In humans, IFN α -based therapy for hepatitis C infection can trigger a major depressive disorder (MDD) resulting in around 30% of patients withdrawing from treatment.^{6,7} Conversely,

Abbreviations: COX, cyclooxygenase; MDD, major depressive disorder; MDMs, monocyte-derived macrophages; SSRI, selective serotonin reuptake inhibitors; TCA, tricyclic antidepressant

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TNF α blockade improves depressive symptoms in patients with rheumatoid arthritis⁸ and in a subgroup of patients with MDD and elevated levels of C-reactive protein (an acute phase protein).⁹

One plausible sequence of events is that peripheral proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6, induced the expression of enzymes involved in tryptophan catabolism, for example, indoleamine dioxygenase, kynurenine hydroxylase, and kynureninase,¹⁰ thereby reducing the availability of synaptic serotonin^{11–13}; which is a proximal cause of depressive symptoms.¹⁴ Inflammation-induced changes in tryptophan metabolism can also lead to increased synthesis of kynurenine and its metabolites, many of which are known to be glutamatergic agonists and/or neurotoxic.¹⁵ Thus, inflammation could produce the “double hit” both reducing the availability of serotonin and increasing production of kynurenine.

The same mechanisms may explain the clinical observation that patients with depressive symptoms in the context of peripheral inflammation (“inflamed depression”) are less responsive to monoaminergic antidepressant drugs, so called “treatment-resistant depression.”¹⁶ Selective serotonin reuptake inhibitors (SSRIs), for example, are thought to increase the synaptic availability of serotonin (by blocking active uptake by presynaptic neurons expressing the serotonin transporter protein (SERT/SLC6A4)).¹⁷ If synaptic serotonin is reduced by an inflammatory response, and SSRIs are not anti-inflammatory,¹⁸ SSRIs are likely to be less effective in the presence of an inflammatory stimulus. Additionally, the neurotoxic effects of kynurenine and its metabolites, which are exacerbated by inflammation, are mediated by glutamatergic-related mechanisms that are not modulated by monoaminergic antidepressants. Treatment-resistant depression—due to reduced serotonin availability and increased neurotoxicity by non-serotonergic mechanisms—is thus predictable in the context of peripheral inflammation.^{12,13,19,20}

Here we report on a series of experiments designed to test the mechanistic connections between inflammatory and monoaminergic systems in a human primary cell model, monocyte-derived macrophages (MDMs). Macrophages are both initiators and mediators of inflammation-associated pathology²¹ and have long been implicated in inflammation induced depression,²⁰ largely based on the inflammatory induced activation of tryptophan depletion pathways.^{10,19,20} Microglia, the macrophages of the brain,²² play a crucial role in neuronal homeostasis, and the impairments in neuronal function associated with many clinical disorders, including major depression.²³ Unlike human macrophages, mouse macrophages do not induce tryptophan uptake or enzymes associated with tryptophan metabolism in response to proinflammatory signals,²⁴ making them a poor model for such studies. In the current study, we have used human MDMs, differentiated by CSF1, to study the impacts of 2 antidepressant drugs and 3 anti-inflammatory drugs on the transcriptional response to 2 proinflammatory challenges: LPS and IFN α . In addition to acting as a primary human cell model for microglia, these cells are also used to examine tryptophan depletion mechanisms during peripheral inflammation, and how drugs may influence this effect.

LPS is a well-studied Gram-negative bacterial cell wall endotoxin that acts through activation of the TLR4 receptor, an archetypal pattern recognition receptor that signals through two well-defined com-

plementary signaling pathways to induce proinflammatory cytokines and IFN target genes.^{25,26} As part of the FANTOM5 consortium, we have recently generated extensive promoter-level data on the time-course of the response of MDM.²⁷ IFN α is a type-1 IFN and was chosen as a stimulus because of the clinical data indicating that this agent can cause a depressive syndrome in patients.^{6,7} We have previously compared and contrasted the transcriptional response of mouse macrophages to type-1 IFN and LPS treatment.²⁸

Three classes of anti-inflammatory drugs were selected to examine their effects on both inflammatory stimuli: a neutralizing antibody for the inflammatory cytokine TNF α ; a nonsteroidal anti-inflammatory drug (indomethacin); and a steroidal anti-inflammatory drug (prednisolone). Neutralizing anti-TNF α antibodies act by binding to TNF α in circulation, thus blocking its proinflammatory effects mediated by action at TNF receptors.²⁹ TNF α acts in an autocrine manner on stimulated macrophages to induce downstream targets and amplify the initial induction of proinflammatory target genes.^{27,30} Indomethacin acts by inhibiting cyclooxygenase (COX) or prostaglandin synthase enzymes (COX1/COX2; PTGS1/PTGS2).³¹ The anti-inflammatory activity of COX inhibitors is thought to depend on preventing inducible prostaglandin production, which can also act in an autocrine manner in macrophages through inducible prostaglandin receptors.²⁵ Both COX-inhibiting and TNF α -inhibiting drugs have been shown to have some antidepressant and anxiolytic effects in patients with psychologic symptoms in the context of medical inflammatory disorders.³² Synthetic glucocorticoids, such as prednisolone, are amongst the most commonly prescribed anti-inflammatory agents, and act by inducing multiple feedback repressors of inflammation, including I κ B and DUSP1.³³ The responses to glucocorticoids also differ radically between humans and mice, due to the gain and loss of glucocorticoid response elements in enhancers,³³ so there is a clear need to study their effects in human systems. The glucocorticoid ligand/receptor complex may also directly repress signaling by interfering with the activation of the inflammatory transcription factor NF- κ B³⁴ leading to a genome wide blockade of NF- κ B interactions with chromatin.³⁵

We also studied the action of two monoaminergic antidepressant drugs on MDMs, a SSRI (escitalopram) and a tricyclic antidepressant (TCA; nortriptyline). SSRIs are the most commonly prescribed drugs for treating depression and their efficacy depends at least partly on increasing synaptic availability of serotonin (5-HT). However, 5-HT also plays an important role in immune signaling,³⁶ and SSRIs have been shown to enhance the cytolytic function of NK cells, to enhance B cell numbers, and to inhibit 5-HT uptake and immune signaling by dendritic cells,³⁷ suggesting that immune mechanisms might also contribute to their therapeutic efficacy. The TCAs are less selectively serotonergic, and also block multiple classes of receptors for acetylcholine, histamine and noradrenaline. The use of TCAs in the treatment of the residual symptoms of inflammatory bowel disease,³⁸ suggests they may have some efficacy as anti-inflammatory agents.³⁹ Supplemental Fig. S1 illustrates the known mode of action of each drug examined here.

We analyzed the gene expression profiles of MDM in response to inflammatory challenge in the presence and absence of

anti-inflammatory or monoaminergic antidepressant drug treatment. Network-based methods were deployed to represent the expression changes induced by inflammatory challenges, and the modulation of response by each of the 5 drugs tested. We tested the specific prior hypotheses (i) that proinflammatory challenges will cause changes in expression of genes related to tryptophan metabolism; (ii) that monoaminergic antidepressants (nortriptyline, escitalopram) will attenuate inflammation-induced changes in tryptophan-related genes; or (iii) that anti-inflammatory drugs (prednisolone, indomethacin, anti-TNF α antibody) will attenuate the regulation of tryptophan-related genes; (iv) that inflammatory activation of human macrophages results in decreased production of tryptophan and increased production of kynurenine; and (v) that anti-inflammatory drugs can attenuate changes in tryptophan metabolism and kynurenine production.

2 | MATERIALS AND METHODS

2.1 | Study design overview

MDM cultures from each of 6 different individuals were treated with 1 of the 3 anti-inflammatory drugs, 2 antidepressant drugs, or a vehicle control. They were then stimulated with either IFN α or LPS for either 7 or 24 h prior to sample collection, or incubated for 24 h with no inflammatory stimulus. Cell culture supernatants were then analyzed by ELISA for cytokine production, and RNA samples extracted from cells and subjected to microarray analysis (Fig. 1). Analysis of these data revealed changes induced by inflammatory challenge in tryptophan/kynurenine gene expression that were moderated by anti-inflammatory drugs and predictive of altered tryptophan/kynurenine metabolism. We tested this prediction in a secondary metabolomics study of the effects of inflammatory challenge and drug treatment on the levels of tryptophan and kynurenine, measured in the cell supernatant using HPLC/MS. A linear mixed effects model was employed with each donor/subject as a random effect to account for interdonor variations, which could potentially lead to false results with a sample size of only $n = 6$ (see below).

2.2 | Ethics and donors

Human CD14⁺ mononuclear cells were isolated from fresh blood of volunteer donors under ethical approval from Lothian Research Ethics Committee (11/AL/0168).

2.3 | Cell culture

Human peripheral blood monocytes were isolated from 320 mL blood samples by Ficoll gradient separation of buffy coats followed by MACS CD14⁺ selection (Miltenyi Biotec Ltd., Biscley, UK). They were then cultured at 5×10^5 cells/well in 1 mL on a 12-well plate in RPMI supplemented with penicillin/streptomycin, glutamax (Invitrogen, Loughborough, UK), and 10% fetal calf serum for 7 d in the presence of rhCSF-1 (a gift from Chiron, Emeryville, CA) at 10^4 U/mL to produce MDM. All donors were medically healthy and between 20 and 50 years old (3 male, 3 female).

IFN α (SRP4594; Sigma-Aldrich, Gillingham, UK) was used at 50 U/mL, within the range reported to be present in blood serum following IFN α -2b/Ribavirin therapy for hepatitis C patients.⁴⁰ LPS from *Salmonella enterica* serotype minnesota (Re 595, L9764; Sigma-Aldrich) was used at 10 ng/mL, which is just maximal for inducible proinflammatory gene expression.²⁸ MDM cultures from each of the 6 individuals were stimulated with IFN α or LPS for 7 or 24 h, or incubated for 24 h with no inflammatory stimulus as a control. Parallel cultures included 100 μ M indomethacin (Sigma-Aldrich), 1 μ M prednisolone (Sigma-Aldrich), 5 μ g IgG1 anti-TNF α antibody (MAB610; R&D Systems, Wiesbaden, Germany), 100 nM escitalopram (Sigma-Aldrich), 1 μ M nortriptyline (Sigma-Aldrich), or IgG1 isotype control (R&D Systems, Abingdon, UK) in DMSO at the times indicated (7 or 24 h). Data supporting the rationale and optimization of inflammatory challenge and drug doses on cell viability assays are provided in Supplemental Materials and Methods. We noted that the dose of both monoaminergic drugs (escitalopram and nortriptyline) was limited by their cytotoxic effects on MDMs in vitro (Supplemental Fig. S2).

2.4 | Optimization of inflammatory stimulation and drug concentrations

Cell viability was measured to optimize the concentrations of drugs used (other than anti-TNF α). This was performed using CellTiter-Glo (Promega Ltd., Southampton, UK) according to manufacturer's instructions. Briefly, cells were seeded at 1×10^5 cells/well in 96-well, flat-bottomed white-walled plates (Corning Ltd., Wiesbaden, Germany) and cultured in 100 μ L media. Following maturation and consequent drug treatment, 100 μ L CellTiter-Glo was added to each well (1:1 ratio) and the plate was placed on a shaker for 10 min. Luminescence was measured in technical triplicates using a GloMax[®] 96- Microplate Luminometer (Promega) and percentage of viable cells after each treatment was calculated relative to the untreated control culture. Six biologic replicates were used to test escitalopram (SSRI) and nortriptyline (TCA), 3 for indomethacin and prednisolone. Supplemental Figs. S2A and B display the cell viability in the presence of escitalopram or nortriptyline at 24 h post-treatment. To ensure maximal drug contact with the cells without affecting viability, concentrations of 100 nM for nortriptyline and 1 μ M for escitalopram were selected for the transcriptomics studies. These concentrations are higher than peak whole blood concentrations of escitalopram (~ 0.1 nM⁴¹) or nortriptyline (50 nM⁴²) in patients. Supplemental Figs. S2C and D show cell viability was not significantly affected by any of the tested concentrations of indomethacin or prednisolone. We therefore selected concentrations of 100 μ M indomethacin and 1 μ M prednisolone, as used in previous in vitro studies of macrophages.^{43,44} The amount of anti-TNF α antibody used was calculated to be 5 μ g/mL. This was based on the suppliers ND₅₀ value of 0.01–0.04 μ g/mL in the presence of 0.75 ng/mL TNF α and our own measurements of maximum TNF α production from stimulated macrophages (<30 ng/mL). We then used the following equation to calculate the amount of antibody required:

$$K_D = \frac{[mAb][Target]}{[Complex]}$$

IFN α (SRP4594, Sigma-Aldrich) was supplemented at 50 U/mL as this concentration yields a robust inflammatory response in

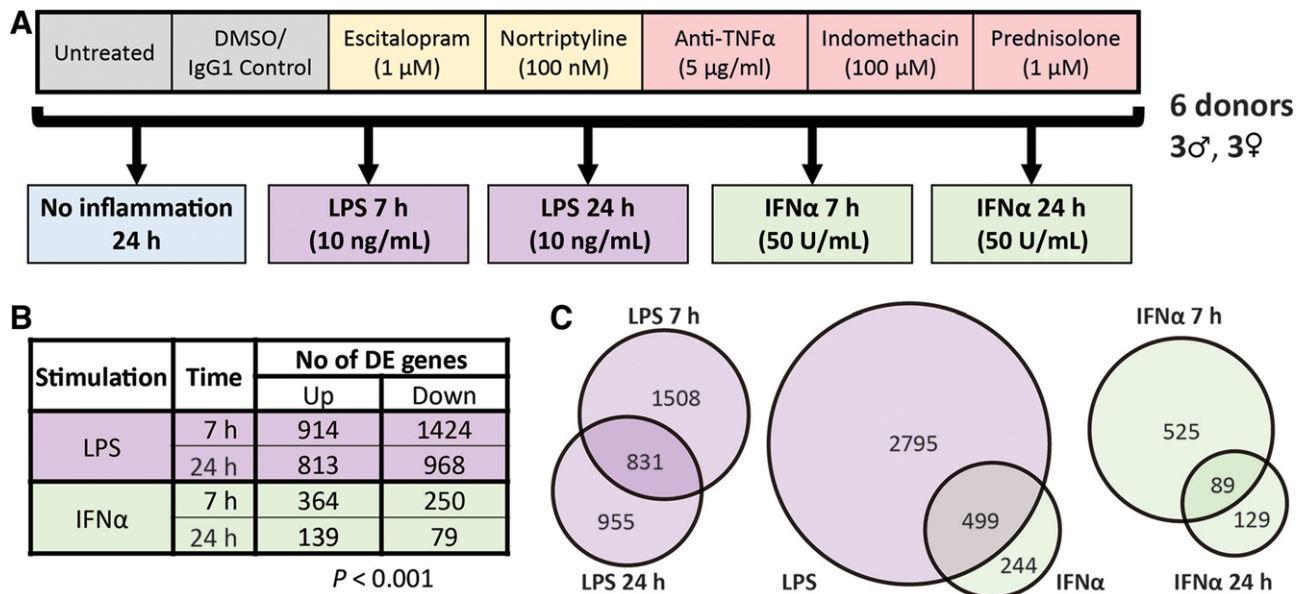


FIGURE 1 Study design and differential gene expression in response to inflammatory challenge. (A) Schematic representation of experimental design. Fully differentiated human MDMs (day 8) were generated from 6 individuals aged 20–30 years, 3 male and 3 female. Cells were then pre-treated with either escitalopram, nortriptyline, an anti-TNF α antibody, indomethacin, prednisolone, or controls (a nonspecific IgG antibody with DMSO vector), or untreated. Cells were then exposed to LPS or IFN α challenge and harvested at either 7 or 24 h, or cultured for 24 h with no inflammatory stimulus. (B) Numbers of significantly differentially expressed genes (DEGs) following inflammatory stimulation ($P < 0.001$). (C) Venn diagrams displaying the overlap of DEGs between IFN α and LPS challenges at early or late time points

macrophages and is within the range reported to be present in blood serum following IFN α -2b/Ribavirin therapy for hepatitis C patients.⁴⁰ Although many in vitro studies typically use 100 ng/mL of LPS, we used 10 ng/mL in order to consistently stimulate the cells for 24 h without obscuring any of the subtler downstream signaling effects (LPS from *S. enterica* serotype minnesota Re 595, L9764; Sigma–Aldrich).

The final parameters for the experiment involving MDM, inflammatory stimuli and drug treatments are outlined in Supplemental Fig. 1A. Each of the MDM cultures from 6 different individuals were treated separately with each of the 3 anti-inflammatory drugs, 2 antidepressants, vehicle control, or left untreated. Samples were then stimulated with either IFN α or LPS for either 7 or 24 h, or incubated for 24 h with no inflammatory stimulus as a control. RNA was extracted from the samples and analyzed by expression microarray

2.5 | RNA extraction and processing

RNA was prepared using RNeasy column-based extraction (Qiagen, Manchester, UK). 350 μ L of RNeasy buffer RLT was used per sample to extract RNA, which was eluted from the column in water following on-column DNase treatment. RNA quality was subsequently analyzed using a 2200 TapeStation (Agilent, Edinburgh, UK). For expression microarrays, 500 ng of RNA was prepared using standard Affymetrix protocols and applied to the Human Gene 2.1 ST array by Edinburgh Genomics (Edinburgh, UK).

2.6 | Expression data analysis

Analysis was performed using R/Bioconductor packages “arrayQualityMetrics,”⁴⁵ “oligo,”⁴⁶ and “nlme.”⁴⁷ Normalization was

performed using RMA.⁴⁸ Probesets were collapsed down to a single gene. These data are available on Gene Expression Omnibus (GEO GSE85333).

Differentially expressed genes (DEGs) at each of the post-inflammatory challenge time points (LPS or IFN at 7 or 24 h) were first estimated by a linear mixed effects model with inflammatory challenge as a fixed effect and donor (participant) as a random effect. To test the hypothesis that drug treatment significantly modulated the genomic response to inflammatory challenge, we extended the linear mixed effects model to include both inflammatory challenge and drug treatment as fixed effects, the interaction between challenge and drug as a fixed effect, and donor as a random effect. This model was fit to data from the second (24 h) post-inflammatory time point. Tests for significance of all linear model coefficients are reported at uncorrected false-positive rates of $P < 0.05$, $P < 0.01$, and $P < 0.001$ as indicated.

Network analysis was performed using Graphia Professional software (Kajeka Ltd., Edinburgh, UK) to explore inflammation- and drug-related transcriptional changes in the context of the transcriptome. Reactome software was used⁴⁹ for enrichment analysis of DEGs previously implicated in neuronal signaling or depression.

2.7 | Quantitation of selected neurotransmitter metabolites

To confirm the predicted metabolic effects of these treatments on the expression of genes related to tryptophan metabolism, tryptophan and kynurenine concentrations were measured by HPLC/MS in the supernatant of cells after 24 h incubation (Supplemental Materials).

2.8 | Quantitation of cytokine production in sample supernatants

To compare protein production to transcript levels, cytokine production was measured in the supernatant of cells by use of TNF- α (#KAC1751) and IL-6 (#KHC0061C) ELISA kits (ThermoFisher, Runcorn, UK). Precoated plates were used according to manufacturer's instructions. Briefly, plates were blocked for nonspecific binding. Supernatants, or standard controls, were then allowed to bind to the precoated antibodies before washing and addition of a secondary HRP-conjugated antibody. Absorbance was read at 450 nm by a plate reader and cytokine concentration was calculated from the standard curve.

3 | RESULTS

3.1 | Transcriptional regulation in MDM by LPS and IFN α

LPS significantly modulated the expression of 3294 genes and IFN α challenge 743 genes in human MDM, consistent with previous observations.^{24,27,50} Both challenges showed the greatest effect at 7 h (Fig. 1 and Supplemental Table S1). As previously observed in mouse macrophages,²⁸ IFN α responsive genes were largely a subset of the LPS response: approximately 2 out of 3 of the IFN α responsive genes were also differentially expressed following LPS treatment. A sample-to-sample correlation network (Fig. 2) demonstrated that samples grouped together according to stimulus. Notably, the 7 h LPS samples were most distant (least correlated with) from the control samples. In keeping with evidence that LPS-inducible genes in monocytes can be treated as quantitative traits with extensive variation between individuals,⁵¹ samples from the same donor tended to be in the same neighborhood.

A network model of the transcriptional network was constructed in which each of 3034 nodes represent a DEG and the edges connecting nodes represented a strong positive correlation ($r > 0.93$) between gene expression profiles across all the samples in the dataset (Fig. 2C). Genes with a similar expression profile tended to group closely together. Regulated genes grouped into 9 major clusters of strongly co-expressed genes: 4 clusters of genes that were up-regulated and 5 clusters of down-regulated genes (Fig. 2D).

3.2 | Effects of monoaminergic antidepressant drugs on whole transcriptome

At the nontoxic doses used in this study, neither antidepressant drug had a significant effect on the macrophage response to either LPS or IFN α (Supplemental Fig. S3).

3.3 | Effects of anti-inflammatory drugs on whole transcriptome

Consistent with their different modes of action, each of the anti-inflammatory drugs influenced the response to LPS or IFN α in a

distinct manner. For example, each drug had a specific effect on the transcription profile of 3 classical inflammatory genes (*IL1B*, *IL6*, and *TNF*) induced by LPS or IFN α challenge (Figs. 3A–F). These data were confirmed at the protein level as measurement of the corresponding cytokine in the supernatants of these same samples (Supplemental Fig. S4) reflected patterns observed at the gene level. A complete list of the DEGs associated with the responses to the two inflammatory stimuli, and the effect of each drug treatment is provided in Supplemental Table S2 and Fig. 3G, respectively. The Venn diagrams (Figs. 3H and I) further illustrate how each drug affected the inflammatory response in a unique manner. Drug modulated genes were visualized in the context of the gene network as a whole (Figs. 3J–L), demonstrating prednisolone and the anti-TNF antibody act predominantly by reducing the expression of genes that were up-regulated, or increasing the expression of genes that were down-regulated by the proinflammatory stimuli. Enrichment analysis confirmed that the genes regulated by LPS or IFN α , and modulated by drug treatment, were functionally important for immune signaling and related to the mechanisms of drug action; see Supplemental Table S1 for a summary and Supplemental Table S3 for details. The effect of indomethacin was more complex than simply inhibiting the responses to LPS and IFN α , consistent with the known ability to prevent expression of prostaglandins, which act as feedback inhibitors of gene expression.⁵² In many cases, it appeared to further elevate the expression of genes already up- or down-regulated, particularly those associated with lipid metabolism.

3.4 | Regulated expression of genes involved in tryptophan metabolism

Amongst the many genes that demonstrated a significant interaction between the two inflammatory stimuli and drug treatment, we focused on pathways for tryptophan catabolism and transport. This pathway relates to the prior hypothesis that tryptophan metabolism could be a key mechanism linking peripheral inflammation to depression.^{10,20,53} A schematic representation of this metabolic pathway is illustrated in Fig. 4.

Both LPS and IFN α caused significant changes in the expression of genes encoding enzyme or transporter proteins that play crucial roles in the metabolism of tryptophan. LPS caused a pronounced up-regulation of genes encoding 2 tryptophan transporters (*SLC16A10* and *SLC7A5*), and up-regulation of genes coding enzymes on the pathway to kynurenine and its metabolites (*IDO1*, *KYNU*, and *KMO*). LPS also caused significant down-regulation of genes coding kynurenine metabolic enzymes (*AFMID* and *CCBL7*). Other tryptophan transporters in human macrophages, the heavy chain Y+L *SLC3A2* (CD98) and *SLC7A7* are expressed constitutively at high levels.²⁷ IFN α challenge resulted in a similar profile of effects on the expression of genes encoding the kynurenine metabolic enzymes, but did not produce significant elevation of the tryptophan transporter genes (Fig. 4).

Anti-inflammatory drugs significantly modulated the on the inducible expression of genes encoding serotonin transporters and kynurenine metabolic enzymes. Interestingly, the direction of change in gene expression caused by anti-inflammatory drug treatment was always opposite in sign to the change caused by activating stimulus.

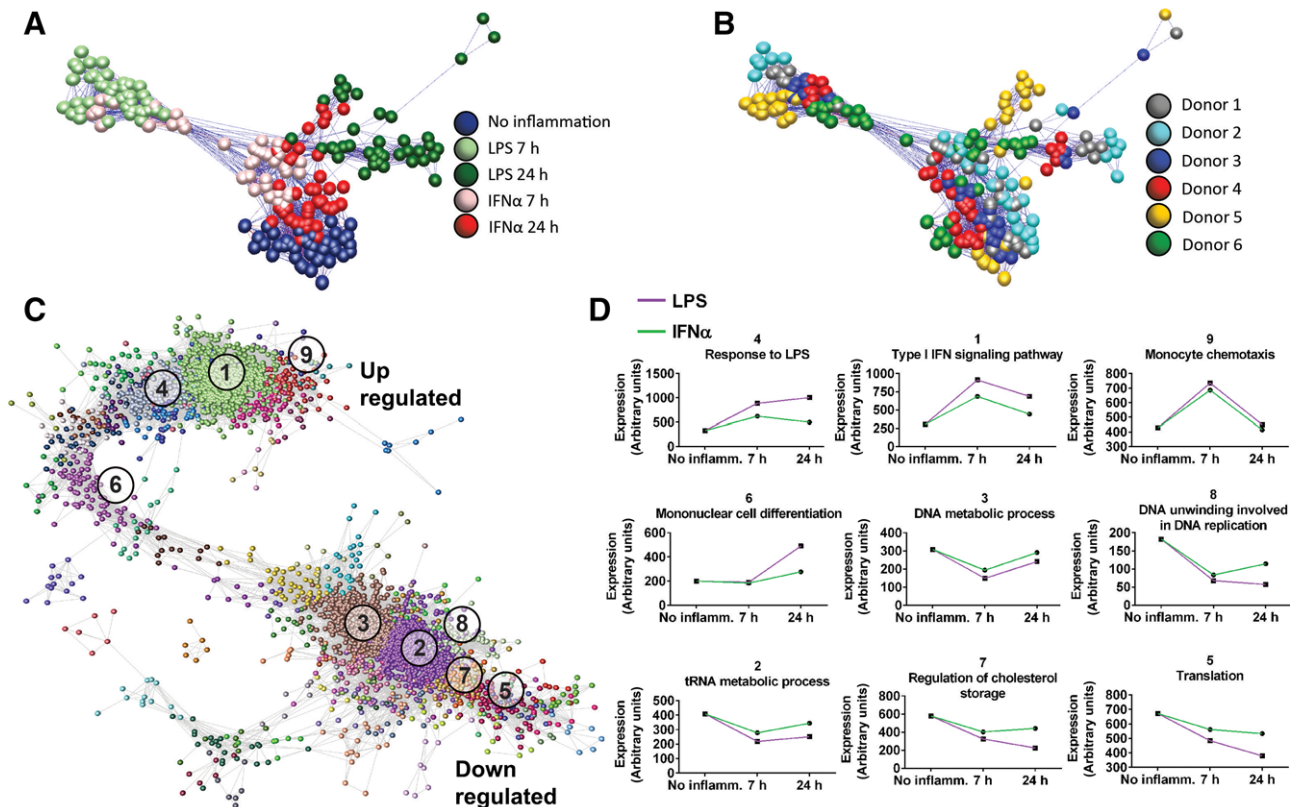


FIGURE 2 Network analysis of the effects of inflammatory challenge and drug treatment on the transcriptome. Sample-sample correlation network constructed from the normalized microarray dataset colored according to (A) inflammatory stimulus or (B) by donor. Nodes represent samples within the dataset and edges represent correlations between samples greater than the threshold ($r > 0.96$). Samples closer to each other are more similar (highly correlated). Separation of the samples is observed by inflammatory stimulus and by donor. (C) A gene-gene correlation network constructed from DEGs from the microarray dataset ($r > 0.93$). Nodes represent genes which cluster together based on their pattern of expression. (D) Mean signal intensity of the genes in 9 of the largest clusters are displayed on the right with GO functional enrichment terms (numbered in order of size)

In other words, 4 of the 5 genes that were up-regulated in response to challenge (*SLC16A10*, *SLC7A5*, *IDO1*, and *KYNU*), were less strongly induced after drug treatments; and all 3 genes that were down-regulated in response to challenge (*SLC7A8*, *AFMID*, and *CCBL7*) were less strongly repressed (Fig. 4).

Thus, all anti-inflammatory drugs tested were found to impact tryptophan- or kynurenine-related gene expression. Indomethacin had effects mainly on tryptophan transporter genes; prednisolone on kynurenine metabolism genes; and anti-TNF α on both class of genes.

3.5 | Effects of macrophage activators (LPS, IFN α) and drugs on tryptophan-related metabolite concentrations

In keeping with the gene expression data, LPS treatment led to 70–80% depletion of tryptophan in the medium and accumulation of kynurenine. IFN α exerted a similar effect, but the magnitude of change in these metabolites was less. Of the three anti-inflammatory drugs tested, only indomethacin significantly attenuated LPS-induced reduction in tryptophan availability (Fig. 5). Both anti-TNF α and indomethacin significantly reduced kynurenine production to ~70% and ~50% following IFN α stimulation but did not reduce kynure-

nine following LPS treatment (Fig. 5). The kynurenine to tryptophan (Kyn/Trp) ratio was used as a measure of tryptophan catabolism overall (Fig. 5). Anti-TNF α treatment significantly moderated IFN α -induced increases in Kyn/Trp ($P = 0.018$); and indomethacin treatment likewise significantly moderated LPS-induced increases in the ratio of kynurenine to tryptophan ($P = 0.026$).

4 | DISCUSSION

Evidence for a link between systemic inflammatory disease and depression (or depressive behavior in animals^{3,5}) is now overwhelming.² We investigated the effects of LPS or IFN α challenge on gene expression by CSF1-cultured MDMs. The scale of the transcriptional response to LPS and IFN α in these data was comparable to previous studies. LPS is itself known to induce type 1 IFNs (mainly IFN β) in MDM, which act in an autocrine manner via IFNAR1, itself induced by LPS.²⁷ Accordingly, the response to exogenous type 1 IFN (IFN α) was largely a subset of the LPS response, as reported in previous studies.^{24,28,50,51,54} None of the anti-inflammatory agents we examined completely prevented the response to either challenge and, although prednisolone and anti-TNF α both reduced expression

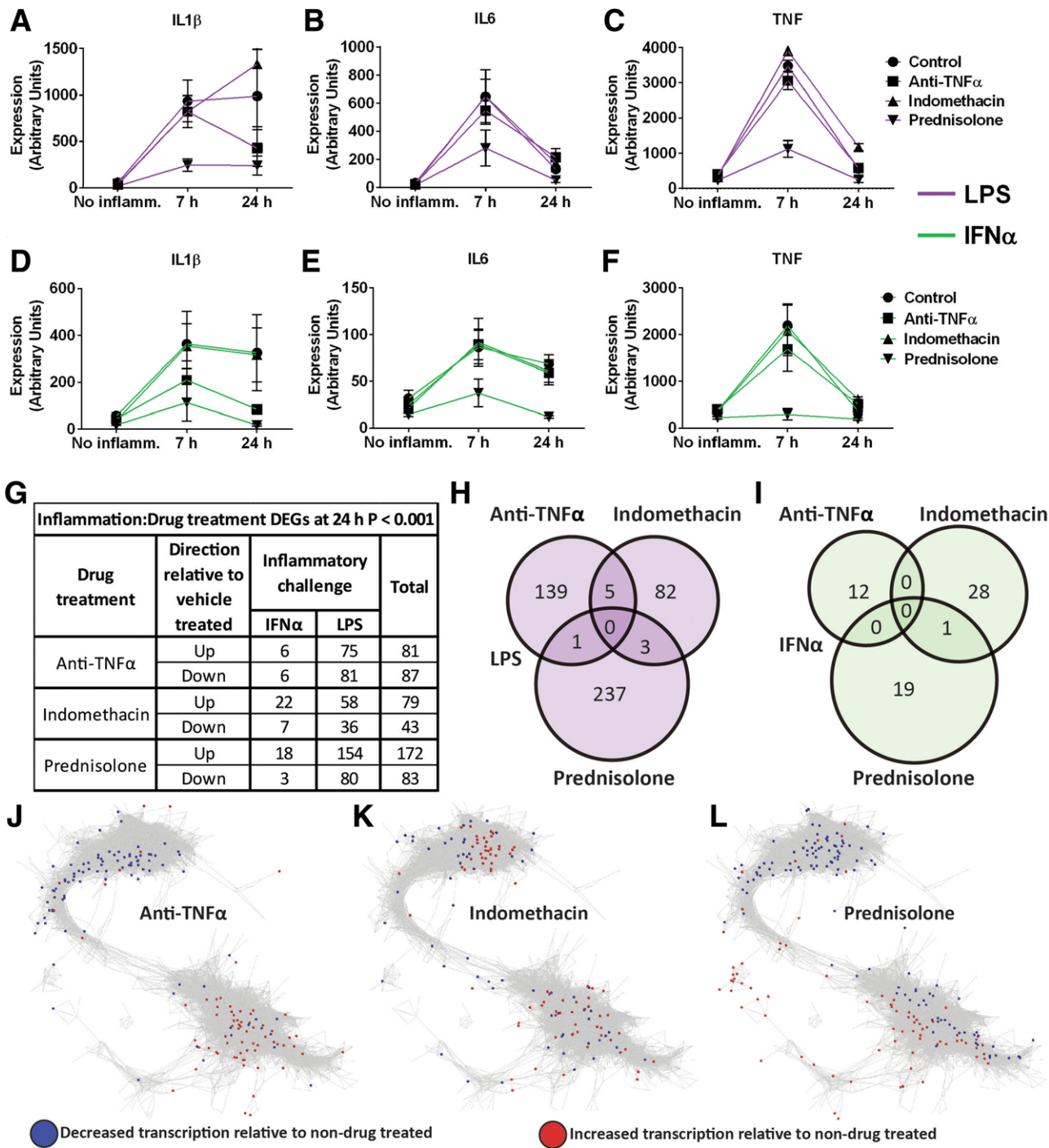


FIGURE 3 Anti-inflammatory drug effects on inflammation-induced gene expression. The expression intensity of *IL1 β* (A, D), *IL6* (B, E), and *TNF* (C, F) are displayed following treatment with anti-inflammatory drugs or vehicle control and LPS (A–C) or IFN α (D–F) challenge. The numbers of genes differentially expressed by the interaction of anti-inflammatory drug treatment and proinflammatory challenge, and their direction of change when compared with control-treated samples, are displayed in a table (G) and as Venn diagrams for each drug (H, I). These DEGs were overlaid on the gene–gene correlation network from Fig. 2: red = genes overexpressed by drug treatment, blue = genes underexpressed by drug treatment. Genes significantly modulated by anti-TNF α treatment (J), indomethacin treatment (K), and prednisolone treatment (L) are displayed separately.

of many genes that were up- or down-regulated by the inflammatory challenges, each had a distinctive transcriptional effect. This diversity likely reflects the complex feed-forward and negative feedback loops that characterize the response to LPS^{27,55} and the different mode of action of each drug.

Both stimuli greatly increased expression (~3000 fold following LPS) of the gene encoding the enzyme indoleamine 2,3-dioxygenase (*IDO1*). *IDO1* catalyzes the primary reaction in conversion of tryptophan to kynurenine; thereby reducing its availability as a precursor for serotonin metabolism. While this process has been

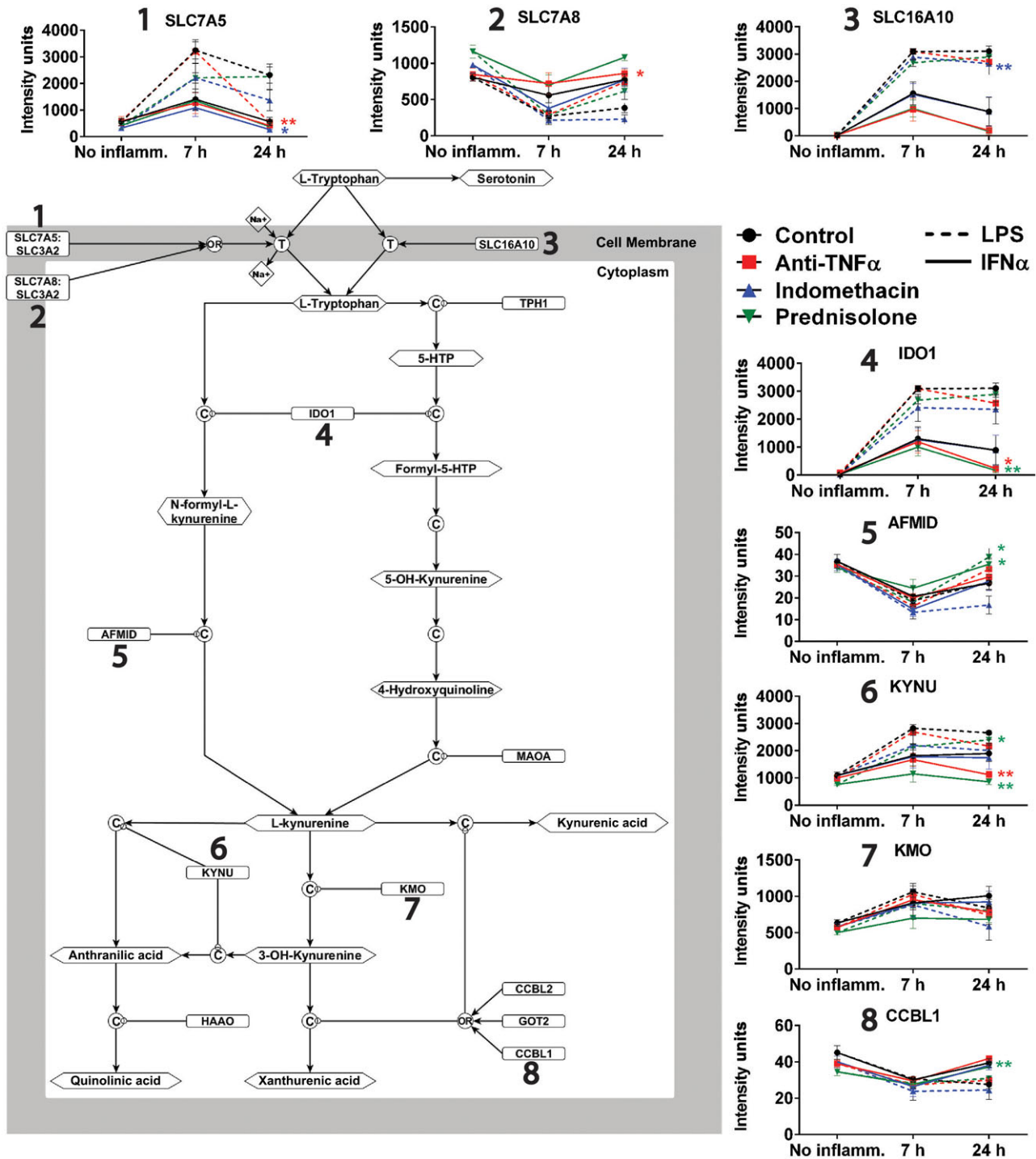


FIGURE 4 Effects of proinflammatory challenge and anti-inflammatory drugs on expression of genes related to tryptophan and kynurenine metabolism. The pathway model summarizes the metabolic role of 8 proteins involved in tryptophan transport or catabolism as illustrated. The profile of expression changes for each gene following proinflammatory challenge is shown by the line graphs during each drug treatment. Significance is depicted for every significant interaction of anti-inflammatory drug treatment with proinflammatory challenge, and color coded for each drug treatment. * $P < 0.05$, ** $P < 0.01$

associated with immunosuppression through Treg activation and effector T cell suppression,⁵⁶ inflammation-induced expression of this enzyme leads to tryptophan depletion both locally and systemically in chronic inflammatory states and has also been linked to alterations in mood.^{5,53,57} Similarly, the ability of host microbiota to control tryptophan metabolism has been functionally linked to influencing mood.⁵⁸

Furthermore, polymorphisms in *IDO1* have been associated with susceptibility to IFN α -induced depression in hepatitis patients.⁵⁹ This may arise both from the depletion of tryptophan, and the generation of neurotoxic metabolites such as kynurenine.^{5,60} However, tryptophan depletion in depressed patients was reportedly independent of kynurenine pathway activation.⁶¹

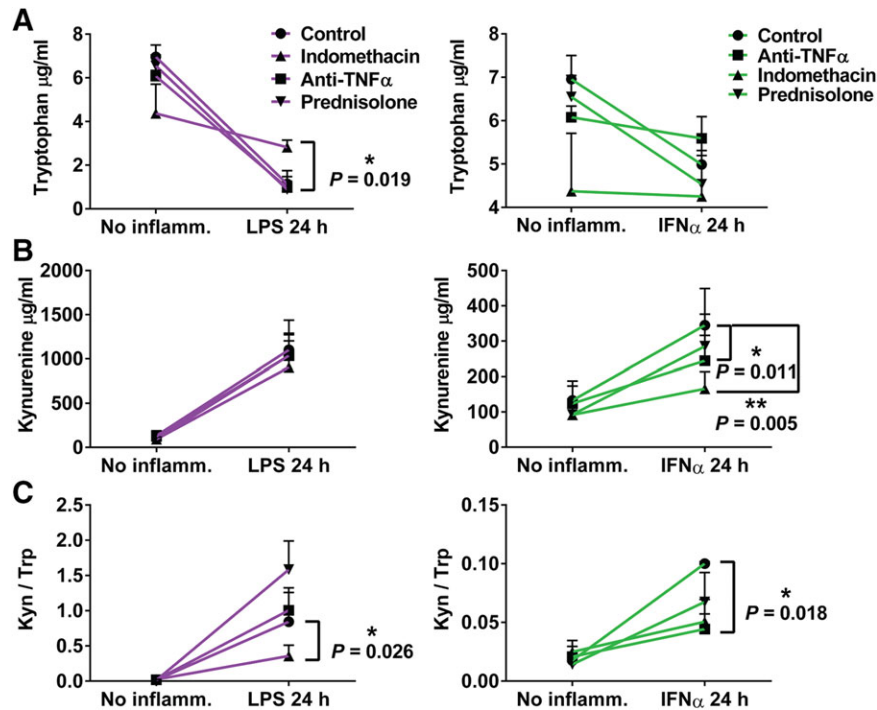


FIGURE 5 Metabolomic analysis of effects of inflammatory challenge and anti-inflammatory drug treatment on tryptophan concentration and tryptophan/kynurenine concentration ratio. Tryptophan (A) and kynurenine (B) concentrations in cell culture supernatants 24 h after pre-treatment of the 3 anti-inflammatory drugs before each inflammatory challenge. (C) Ratio of kynurenine to tryptophan levels detected in the supernatant. Significance was calculated using a ratio paired *t* test. **P* < 0.05 ***P* < 0.001

Anti-TNF α reduced transcription of tryptophan catabolism enzymes, *IDO1* and *KYNU*, following IFN α stimulation but no significant effect of the drug was observed on these elements following LPS treatment. This is likely due to the transient induction of feed-forward and negative feedback loops of TNF α transcription following inflammatory stimulus. Like IFN α , TNF α was predicted to be involved in an autocrine loop in MDM elicited by LPS, since the TNF α receptor is also induced.²⁷ Our data (Supplemental Table S2) confirm and extend evidence of autocrine TNF α signaling as a feed-forward activator of macrophage gene expression^{30,62,63} and identify the subset of inducible genes dependent upon that stimulus. The lack of impact of anti-TNF α on LPS-inducible *IDO1* may reflect the magnitude of the response. Alternatively, activation of *IDO1* by IFN- γ requires co-stimulation by TNF α , which increases the occupancy of IFN-response elements.^{64,65} It may be that LPS provides this second signal independently of TNF α .^{30,62,63}

A study examining the treatment of primary murine hippocampal cells with ibuprofen, a nonselective COX inhibitor like indomethacin, identified *TDO2* as the most significantly affected gene.⁶⁶ *TDO2*, which is not expressed in macrophages, acts like *IDO1* to metabolize tryptophan to kynurenic metabolites. However, although anti-TNF α and prednisolone each significantly reduced *IDO1* activity at 24 h post-IFN α stimulation, indomethacin treatment did not. Indomethacin did, however, significantly reduce transcription of tryptophan transporters induced by both inflammatory challenges, potentially reducing the availability of intracellular tryptophan as a substrate for *IDO1*. In contrast, anti-TNF α treatment reduced transcription of one trypto-

phan transporter while increasing transcription of another during LPS challenge, and prednisolone did not affect the mRNA levels of any transporter.

To directly assess tryptophan uptake and catabolism following each treatment, we measured the concentrations of tryptophan and kynurenine in the supernatant. Indomethacin was the only inhibitor that impacted tryptophan levels during LPS challenge, likely due to a transcription repression of the tryptophan transporter. Prednisolone, while modulating expression of numerous inducible genes, did not produce any alteration in overall tryptophan catabolism (Supplemental Fig. S5), despite reducing expression of tryptophan catabolism-related genes such as *IDO1*, *KYNU*, and *CCBL1*.

A recent review examined the anti-inflammatory effects of antidepressant drugs.⁶⁷ We found no evidence that either of the monoaminergic antidepressant drugs tested (TCA or SSRI) exerted any effect on the response in MDMs to LPS or IFN α . This is in contrast to reports that TCAs reduce proinflammatory signaling in phagocytes,⁶⁸ and SSRIs have been reported to alter macrophage differentiation and inflammatory signaling.^{67,69} SSRIs have also been reported to modulate glucocorticoid actions on monocytes.⁷⁰ Although we found no direct effect of SSRIs or TCAs on macrophage responses to either LPS or IFN α , we did observe significant cytotoxicity at concentrations that were <20-fold lower than circulating blood concentrations in treated patients. In support of this, nortriptyline has previously been shown to induce autophagy in macrophages.³⁹ Indeed, many antidepressants are cationic amphipaths, and are therefore

lysosomotropic⁷¹ likely accumulating selectively in phagocytic cells. Selective toxicity to macrophages may therefore contribute to the results reported in other studies, which used far higher concentrations of SSRIs than used here.⁶⁹

There is already some evidence that anti-inflammatory drugs can have antidepressant efficacy.³² Our results are compatible with the mechanistic interpretation that this may be at least partly attributable to the effects of anti-inflammatory drugs on "normalization" of an inflammation-induced bias in tryptophan and kynurenine metabolism. However, the results also indicate that this is unlikely to be the sole mechanism, and each agent may produce distinct patterns of regulation of genes that can impact indirectly on neuronal function expression. If each anti-inflammatory agent is distinct in its actions, it may be that combinations would have novel potential efficacy.

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DISCLOSURE

E.T.B. is employed half-time by the University of Cambridge and half-time by GSK; he holds stock in GSK.

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